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Change of blue ink margin colour to black in immunohistochemistry stained slides: a potential pitfall for pathology cancer margin assessment

Sir,

In the course of our clinical practice we have become aware that some inks applied to tissue margins may change colour after immunohistochemical (IHC) staining. In evaluating this occurrence systematically across multiple breast cancers, different IHC stains, and two different IHC platforms at two different IHC laboratories, we have observed fidelity of the ink colour during routine processing and on H&E sections but a consistent change in the colour of the blue ink to black occurs after IHC staining. Awareness of this artefact of IHC processing will assist pathologists avoid a potentially serious pitfall with significant implications for patient care.

For invasive breast carcinoma, the finding of tumour at an inked margin defines a positive margin,¹ while for women with ductal carcinoma *in situ* treated with breast conserving surgery (BCS) with whole breast irradiation, a minimum clearance of 2 mm is recommended.² Because positive surgical margins are the most important modifiable risk factor for local recurrence of breast cancer, further surgery is often undertaken in an attempt to achieve acceptable margins. Surgeons and pathologists orient specimens in three dimensions to permit assessment of each surgical margin. Inking helps identify true margins on histology, while the application of a different colour indelible ink to each margin allows pathologists to specify the anatomical location of involved margins, so re-excisions may be targeted appropriately.

When epithelial proliferations occur close to margins, IHC may be helpful in distinguishing malignant lesions from benign processes. In the course of our clinical experience, we have become aware that some inks change colour after IHC staining. This colour change may lead to the erroneous anatomical designation of the involved margin, potentially impacting decisions regarding further surgery. In this brief report we wish to highlight this potential pitfall, to be mitigated by pathologists.

Following the identification of an index case, we systematically evaluated ink colour preservation in breast specimens from its application to the gross specimen, through routine processing, H&E morphology and IHC, using two different IHC platforms. Seventeen clinical breast samples and three additional composite blocks created for this quality assurance purpose were assessed.

We use blue, green, black, orange, red and yellow inks manufactured by the Davidson Marking System (DMS; Trajan Scientific and Medical, Australia). DMS inks are widely used in histology laboratories. For clinical samples,

breast cancer resections with corresponding IHC slides were included, if the IHC slides included margins inked with different colour dyes.

For further quality assurance evaluation, we prepared three composite blocks of surplus breast tissue, each comprised of three strips of cancer and benign tissue. The two long surfaces of each strip were painted with different inks, such that the three strips in each block included all six ink colours. These blocks were processed routinely for H&E assessment. Routine IHC evaluation was carried out for P63 or CD45 at two laboratories, one using the Ventana platform with the ultraView Universal DAB detection kit (product code 760-500; Ventana, USA), and the other the Dako Autostainer Link 48 IHC platform (Dako, Denmark), with the Ultravision LP detection system (product code TL-125-HL; Thermo Scientific, USA) and Dako Liquid DAB/substrate chromagen system (product code K3468; Dako). We recorded the colours of the inks at the edges of the tissue at each step in the process, including at the time of prosection, in the processed block, the H&E section and on the corresponding IHC slide.

Per standard protocols, quality assurance evaluations are exempt from formal evaluation through the institutional human research ethics committee.

The index case was an invasive breast cancer which included an atypical epithelial proliferation at the blue inked superior margin, necessitating IHC evaluation to distinguish invasive carcinoma from adenosis. IHC for P63 showed absence of a myoepithelial cell layer, confirming the impression of invasive carcinoma. When reviewed for a multidisciplinary meeting, it was noted that the involved margin was black. In this case black ink had been applied to the deep margin. This distinction was critical for management as our surgeons frequently leave no breast tissue at the superficial and deep aspects of their wide local excisions for breast re-excisions to be attempted, whereas cancer at the four circumferential margins is an indication for further breast surgery. After careful correlation with the H&E sections, it was determined that the lesion was indeed at the superior margin and the reason for the discrepancy was a change of ink colour from blue to black during IHC staining.

In our formal analysis of 20 further samples, we found fidelity of the ink colours through routine processing and H&E staining. However, in all 20 cases analysed, the blue ink changed colour to black following IHC (Fig. 1). This was observed at two IHC laboratories, on the Ventana and the Dako platforms and independently of the antibody used. The remaining ink colours were maintained after IHC processing.

Histological assessment of surgical margins of cancer resections is central to clinical care. Depending on the precise anatomical location of the margin, positive margins may be an indication for further surgery. The extent of disease at margin and margin of clearance may also influence decisions regarding radiation therapy.

Various difficulties have been described in the assessment of breast surgical margins.³ These include sampling errors, inadvertent inking of non-marginal surfaces and difficulties in distinguishing different colours, such as the orange and red ink combination.⁴ The observation of a consistent change of the blue ink colour to black following IHC staining signifies a potentially serious pitfall. Apart from the colour change, the tissue reveals no structural or tinctorial clues to alert the pathologist, such that without prior knowledge of this

Fig. 1 (A) H&E section showing breast carcinoma at a blue inked margin. (B) Corresponding AE1/AE3 IHC slide showing change of the blue dye colour to black.

possibility, the anatomical designation of an involved margin may be incorrect.

Margin re-excision specimens may be particularly problematic if the blue and black ink combination are used to mark the old and new margins. Often only two colours are used in these specimens and the shape of the tissue has little variation to assist with orientation.

It is particularly unfortunate that the problem is specific to the blue ink and results in its change to black, since these two colours are preferred by pathologists when scored for discernment of colour, intensity, and consistency of coverage⁵ and these colours are not subject to misinterpretation caused by red green colour blindness which affects 8% of males and 0.5% of females.

We have found no prior reference to this problem in the breast pathology literature. Williams *et al.* did describe this phenomenon in more general terms, when tissue was subjected to prolonged decalcification as well as H&E and IHC.⁶ They also saw the blue and the violet inks from DMS change to black after IHC. None of the samples in our study were decalcified. In all cases, the surgical records verify that the tissue was placed in formalin promptly after excision and certainly within less than 1 hour of cold ischaemic time recommended by the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) and endorsed by the Royal College of Pathologists of Australasia for optimal biomarker assessment.

Since becoming aware of this potential pitfall and carrying out a formal audit in the Ventana platform with the ultraView Universal DAB detection kit, we have found that a blue dye by a different manufacturer (CDI REF0728-2; Cancer Diagnostics Inc, USA) is colour-fast after IHC staining and we now use this product routinely.

We highlight a consistent colour change in the DMS tissue marking blue ink to black after IHC staining, independent of IHC platforms. Awareness of this pitfall may prevent potentially serious errors in anatomical designation of involved margins by pathologists.

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Understanding and using journal metrics: seeing past the numbers

Sir,

The Editorial by Delahunt *et al.*, asks the question ‘Publication metrics: what do they mean?’ and, after a discussion of the Journal Impact Factor (JIF) and CiteScore, dismisses publication metrics generally by answering the question posed with ‘not much’.¹ The meaning of any metric, I would argue, is not inherent in the metric, but dependent on the context in which it is presented and the purpose to which it is